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Doxycycline Is Anti-Inflammatory and Inhibits Staphylococcal Exotoxin-Induced Cytokines and Chemokines

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Proinflammatory cytokines mediate the toxic effect of superantigenic staphylococcal exotoxins (SE). Doxycycline inhibited SE-stimulated T-cell proliferation and production of cytokines and chemokines by human peripheral blood mononuclear cells. These results suggest that the antibiotic doxycycline has anti-inflammatory effects and is therapeutically useful for mitigating the pathogenic effects of SE.

Staphylococcal toxic shock syndrome toxin 1 (TSST-1) and the structurally related exotoxins are bacterial exotoxins that bind directly to major histocompatibility complex class II molecules on antigen-presenting cells (1, 5, 8, 18, 23) and activate T cells expressing specific Vβ elements (7). These toxins are called superantigens because of their ability to polyclonally stimulate large populations of T cells (1, 4, 7, 14). Thus, staphylococcal exotoxins (SE) are potent activators of the immune system and cause a variety of diseases in humans, including food poisoning, toxic shock, and autoimmune diseases (1, 2, 6, 12, 14, 22). Their interactions with cells of the immune system result in massive production of proinflammatory cytokines and chemokines (1, 4, 15, 17). The cytokines tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and gamma interferon (IFN-γ) are key mediators in superantigen-induced toxic shock (1, 21). Both TNF-α and IL-1 have potent immunostimulating activities and act synergistically with IFN-γ to enhance immune reactions and promote tissue injury (16). Consequently, these cytokines are pathogenic at high concentrations in vivo and are responsible for fever and toxic shock induced by SE (13, 14, 18, 19).

Doxycycline is a broad-spectrum antibiotic widely used for infections caused by both gram-negative and gram-positive microorganisms. It acts as a bacteriostatic agent and is highly effective against many microorganisms, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus anthracis*, and *Yersinia pestis*. Doxycycline belongs to the tetracycline antibiotic family, the members of which have been shown to have other biological actions independent of their antimicrobial effects (10). Doxycycline inhibits phorbol-12-myristate-13-acetate-mediated matrix metalloproteinase 8 (MMP-8) and MMP-9 in human endothelial cells (11). Doxycycline also decreases elastin degradation and reduces MMP activity in a model of aneurismal disease (3). More recently, doxycycline was shown to inhibit the production of IL-1β in lipopolysaccharide-treated corneal epithelial cultures to an extent comparable to that achieved by corticosteroids (25). In vivo, doxycycline protected mice from lethal endotoxemia by downregulating cytokine and nitrate secretion in blood (20). This study was undertaken to determine the modulatory effect of doxycycline on staphylococcal superantigen-induced T-cell activation and cytokine production from human peripheral blood mononuclear cells (PBMC).

Purified SEB and TSST-1 were obtained from Toxin Technology (Sarasota, Fla.). The endotoxin content of these preparations was <1 ng of endotoxin/mg of protein, as determined by the *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, Md.). Human recombinant TNF-α (hTNF-α), antibodies against hTNF-α, peroxidase-conjugated anti-rabbit immunoglobulin G, and peroxidase-conjugated anti-goat immunoglobulin G were obtained from Boehringer Mannheim (Indianapolis, Ind.). Recombinant monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and antibodies against hIL-1β, hIL-6, hMIP-1α, and MIP-1β were purchased from R&D Systems (Minneapolis, Minn.). Human rIL-1β was kindly provided by J. Oppenheim (National Cancer Institute, Frederick, Md.). Human recombinant IFN-γ (rIFN-γ) and rIL-6 were obtained from Collaborative Research (Boston, Mass.). Antibodies against hIFN-γ and MCP-1 were obtained from Pharmingen (San Diego, Calif.). Doxycycline was purchased from Sigma (St. Louis, Mo.) and dissolved in phosphate-buffered saline, pH 7.4. All other reagents were also from Sigma.

Human PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal human donors. PBMC (10^6/ml) were cultured at 37°C in 24-well plates containing RPMI 1640 medium and 10% heat-inactivated fetal bovine serum. Cells were incubated with either SEB (200 ng/ml) or TSST-1 (200 ng/ml) for 16 h, and the supernatants were harvested and analyzed for IL-1β, TNF-α, IL-6, IFN-γ, MCP-1, MIP-1α, and MIP-1β. Cytokines and chemokines were measured by an enzyme-linked immunosorbent assay with cytokine- or chemokine-specific antibodies in accordance with the manufacturer's instructions (15, 17). Human recombinant cytokines and chemokines (20 to 1,000 pg/ml) were used as standards for calibration on each plate. The detection limit of each assay was 20 pg/ml. The cytokine and chemokine data were expressed as the mean reading ± the standard deviation (SD) of duplicate samples. Doxycycline, when present, was added simultaneously with the stimulating agent. Cytotoxicity

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14. ABSTRACT

Proinflammatory cytokines mediate the toxic effect of superantigenic staphylococcal exotoxins (SE). Doxycycline inhibited SE-stimulated T-cell proliferation and production of cytokines and chemokines by human peripheral blood mononuclear cells. These results suggest that the antibiotic doxycycline has anti-inflammatory effects and is therapeutically useful for mitigating the pathogenic effects of SE.

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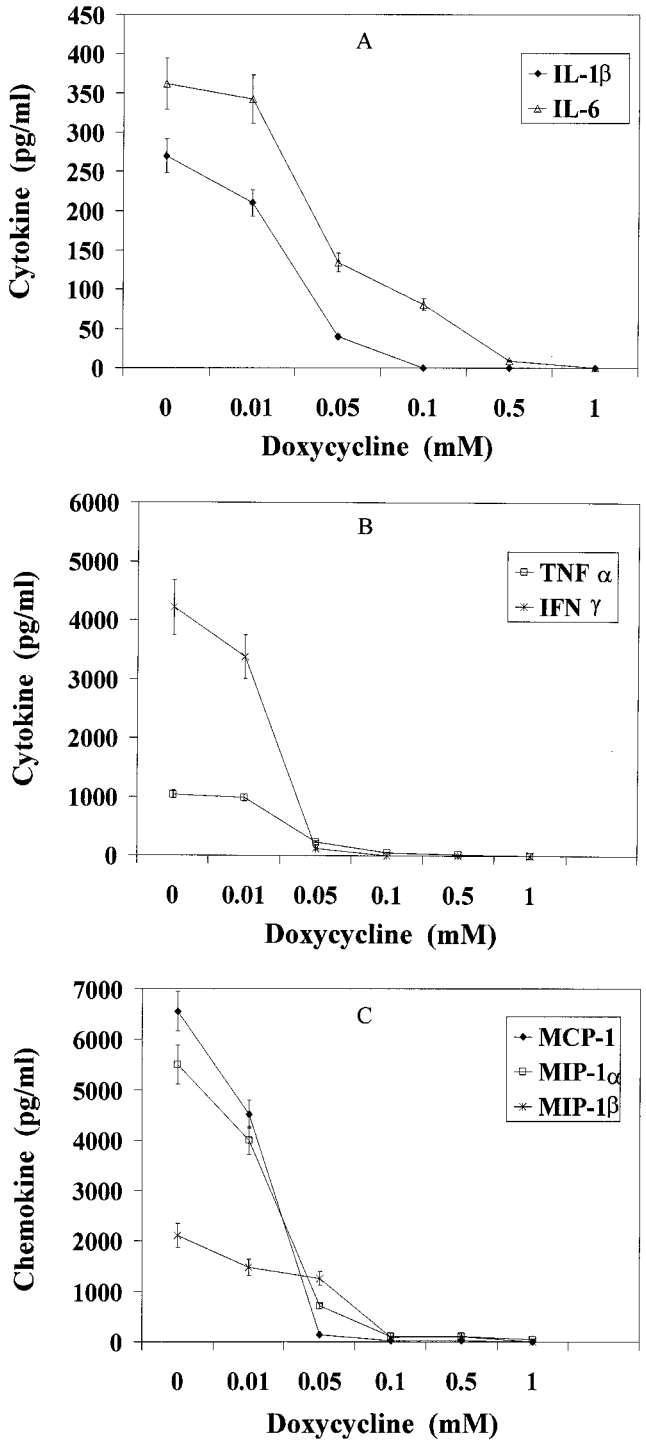

FIG. 1. Dose-response inhibition of IL-1β and IL-6 (A), TNF-α and IFN-γ (B), and MCP-1, MIP-1α, and MIP-1β (C) production by PBMC stimulated with 200 ng of SEB per ml in the presence of various concentrations of doxycycline. Values represent the mean ± SD of duplicate samples from three experiments.

was measured by the release of lactate dehydrogenase (LDH) from the cytosol into culture supernatant. LDH was quantitated by using a colorimetric cytotoxicity assay kit (Boehringer Mannheim) as instructed by the manufacturer. The maximum amount of releasable LDH (100%) was obtained by lysing cells with 1% Triton X-100. T-cell proliferation was assayed with PBMC (10^5/well), which were plated in triplicate with SEB or TSST-1 (200 ng/ml), with or without doxycycline, for 48 h at 37°C in 96-well microtiter plates. Cells were pulsed with 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.) per well during the last 5 h of culture as described previously (15). Cells were harvested onto glass fiber filters, and incorporated [^{3}H]thymidine was measured by liquid scintillation. All data were analyzed for significant differences by Student's *t* test with Stata (Stata Corp., College Station, Tex.). Differences between doxycycline-treated and untreated control groups were considered significant if P was <0.05.

On the basis of the report that doxycycline blocked lipopolysaccharide-induced IL-1 in epithelial cells and prevented lethal endotoxemia in vivo (20, 25), we tested the hypothesis that this antibiotic might have direct effects on SE-induced cytokines. As shown in Fig. 1, doxycycline dose dependently inhibited the production of the cytokines IL-1β, IL-6, TNF-α, and IFN-γ and the chemokines MCP-1, MIP-1α, and MIP-1β by PBMC incubated with SEB. Similar dose-dependent reduction of cytokines and chemokines by doxycycline was also observed for TSST-1-stimulated PBMC (data not shown). The inhibitory effect of doxycycline on SEB- or TSST-1-mediated cytokines and chemokines obtained with PBMC from seven normal donors is summarized in Fig. 2. Production of MCP-1 and IFN-γ was completely blocked by 50 μM doxycycline. This concen-

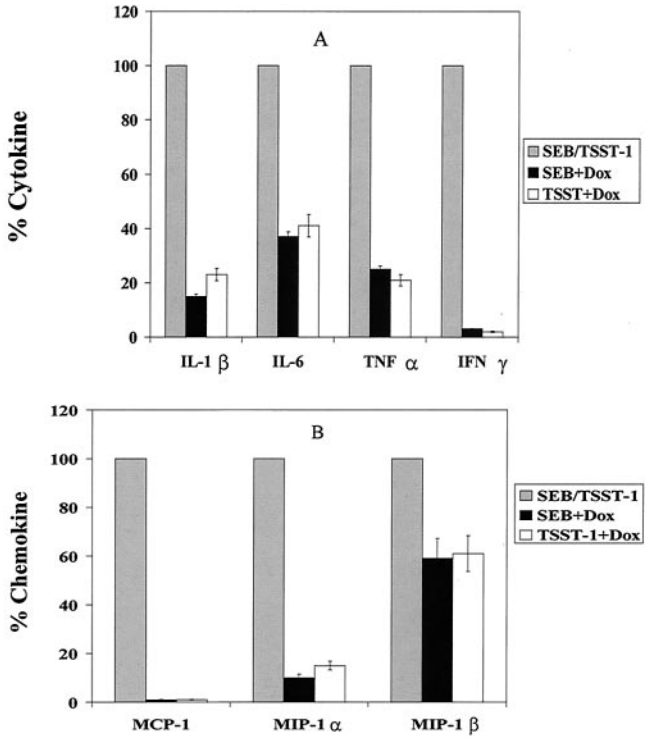

FIG. 2. Inhibition of IL-1β, IL-6, TNF-α, and IFN-γ (A) and MCP-1, MIP-1α, and MIP-1β (B) production by PBMC stimulated with SEB (200 ng/ml) or TSST-1 (200 ng/ml) in the presence of 0.05 mM doxycycline (Dox). Values represent the mean ± SD of PBMC cultures from seven blood donors. Results are statistically significantly different ($P < 0.05$) between SE and SE-plus-doxycycline samples.

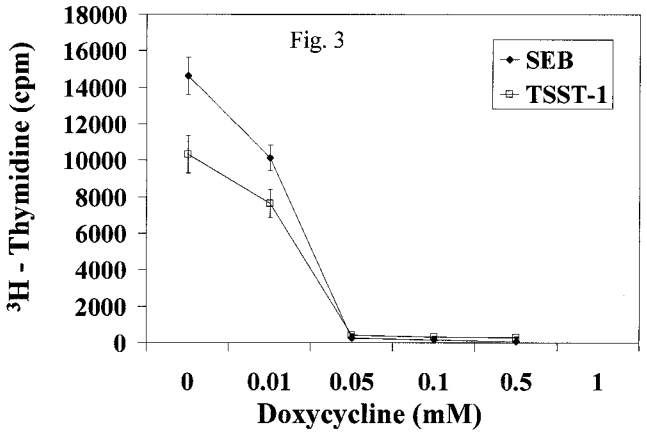

FIG. 3. Inhibition of T-cell proliferation in PBMC stimulated with 200 ng of SEB or TSST-1 per ml by various concentrations of doxycycline. Values are the mean count ± the standard error of the mean of triplicate cultures and represent five experiments. Results are statistically significantly different ($P < 0.02$) between SE and SE-plus-doxycycline samples.

tration of doxycycline reduced IL-1β, IL-6, TNF-α, MIP-1α, and MIP-1β to 15 to 22%, 37 to 41%, 21 to 25%, 10 to 15%, and 59 to 61% of that of untreated, SEB- or TSST-stimulated cells, respectively. TNF-β, when present, was also inhibited to 25% of that of untreated, SEB-stimulated cells. Doxycycline was not cytotoxic to PBMC at this concentration as measured by the exclusion of trypan blue and the lack of lactate dehydrogenase release from treated cells. Complete inhibition of these cytokines and chemokines was observed at high doses of doxycycline (>0.1 mM). Similar dose-response inhibition by doxycycline was observed at lower concentrations of SEB (1 and 10 ng/ml) (data not shown).

Because superantigens also cause T-cell proliferation, the effect of doxycycline on SE-induced T-cell proliferation was investigated. Figure 3 shows that doxycycline inhibited SEB- and TSST-1-stimulated T-cell proliferation in a dose-dependent manner, achieving 98% inhibition at 0.05 mM.

This study demonstrated that doxycycline effectively inhibited superantigen-mediated production of cytokines and chemokines by human PBMC in vitro. T-cell proliferation induced by staphylococcal superantigens was also suppressed completely. Downregulation of proinflammatory cytokines and chemokines by doxycycline in SEB- and TSST-1-stimulated PBMC suggested that doxycycline may affect the pathophysiology of toxic shock. These findings extend the observations of other investigators of the immunomodulatory effects of doxycycline in addition to its antimicrobial activities.

Multiple molecular mechanisms, both transcriptional and posttranscriptional, may be involved in the anti-inflammatory effects of doxycycline (11, 26). The suppression of proinflammatory cytokines may involve the downregulation of the PKC pathway by doxycycline, as suggested by a study of its effects on granuloma formation (26). The reported inhibitory dose of doxycycline (10 to 15 μM) that reduces collagenase, gelatinase, and other metalloproteinases in vitro (10, 11) is comparable to that used in this study and is severalfold higher than that observed in human serum after oral dosing of 200 mg daily (10, 24). However, clinical studies indicate that this dose was sufficient in reducing the collagenase and gelatinase activities in human osteoarthritic cartilage extracts ex vivo (24). A subantimicrobial dose of doxycycline (20 mg twice daily) has been shown to inhibit gingival fluid collagenase activity (9). In addition, in vivo studies of experimental endotoxemia also found doxycycline and other tetracyclines efficacious in downregulating inflammatory cytokines and preventing shock (20).

In conclusion, the results presented here indicate that doxycycline down-regulates proinflammatory cytokines and chemokines, thus suggesting its potential utility for treating superantigen-induced toxic shock. In a clinical setting when the host is exposed to multiple biological agents, including both bacteria and bacterial exotoxins, the use of doxycycline offers an additional advantage of providing both antimicrobial and anti-inflammatory effects.

The views expressed in this publication are those of the authors and do not reflect the official policy or position of the Department of the Army, the Department of Defense, or the U.S. Government.

REFERENCES

1. **Baker, M. D., and K. A. Acharya.** 2003. Superantigens: structure, function, and diversity, p. 1–31. *In* T. Krakauer (ed.), Superantigen protocols. Humana Press, Totowa, N.J.
2. **Bergdoll, M. S., and P. M. Schlievert.** 1984. Toxic shock syndrome toxin. Lancet **ii:**691.
3. **Boyle, J. R., E. McDermott, M. Crowther, A. D. Wills, P. R. Bell, and M. M. Thompson.** 1998. Doxycycline inhibits elastin degradation and reduces metalloproteinase activity in a model of aneurismal disease. J. Vasc. Surg. **27:**354–361.
4. **Cameron, S. B., M. C. Nawijn, W. W. Kum, H. F. Savelkoul, and A. W. Chow.** 2001. Regulation of helper T cell responses to staphylococcal superantigens. Eur. Cytokine Netw. **12:**210–222.
5. **Chatila, T., and R. S. Geha.** 1993. Signal transduction of microbial superantigens via MHC class II molecules. Immunol. Rev. **131:**43–59.
6. **Chesney, P. J., J. P. Davis, W. K. Purdy, P. J. Wand, and R. W. Chesney.** 1981. Clinical manifestations of toxic shock syndrome. JAMA **246:**741–748.
7. **Choi, Y., B. Kotzin, L. Hernon, J. Callahan, P. Marrack, and J. Kappler.** 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. Proc. Natl. Acad. Sci. USA **86:**8941–8945.
8. **Gascoigne, N. R. J., and K. T. Ames.** 1991. Direct binding of secreted TCR β chain to superantigen associated with MHC class II complex protein. Proc. Natl. Acad. Sci. USA **88:**613–616.
9. **Golub, L. M., T. F. McNamara, M. E. Ryan, B. Kohut, T. Blieden, G. Payonk, T. Sipos, and H. J. Baron.** 2001. Adjunctive treatment with subantimicrobial doses of doxycycline: effects on gingival fluid collagenase activity and attachment loss in adult periodontitis. J. Clin. Periodontol. **28:**146–156.
10. **Golub, L. M., N. S. Ramamurthy, and T. F. McNamara.** 1991. Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs. Crit. Rev. Oral Biol. Med. **2:**297–322.
11. **Hanemaaijer, R., H. Visser, P. Koolwijk, T. Sorsa, T. Salo, L. M. Golub, and V. W. Hinsbergh.** 1998. Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. Adv. Dent. Res. **12:**114–118.
12. **Holmberg, S. D., and P. A. Blake.** 1984. Staphylococcal food poisoning in the United States: new facts and old misconceptions. JAMA **251:**487–489.
13. **Huang, W. T., M. T. Lin, and S. J. Won.** 1997. Staphylococcal enterotoxin A-induced fever is associated with increased circulating levels of cytokines in rabbits. Infect. Immun. **65:**2656–2662.
14. **Kotzin, B. L., D. Y. M. Leung, J. Kappler, and P. A. Marrack.** 1993. Superantigens and their potential role in human disease. Adv. Immunol. **54:**99–166.
15. **Krakauer, T.** 1994. Inhibition of toxic shock syndrome toxin-induced cytokine production and T cell activation by interleukin 10, interleukin 4, and dexamethasone. J. Infect. Dis. **172:**988–992.
16. **Krakauer, T., J. Vilcek, and J. J. Oppenheim.** 1998. Proinflammatory cytokines: TNF and IL-1 families, chemokines, TGFβ and others, p. 775–811. *In* W. Paul (ed.), Fundamental immunology, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa.
17. **Krakauer, T.** 1999. Induction of CC chemokines in human peripheral blood mononuclear cells by staphylococcal exotoxins and its prevention by pentoxifylline. J. Leukoc. Biol. **66:**158–164.
18. **McCormick, J. K., J. M. Yarwood, and P. M. Schlievert.** 2001. Toxic shock

syndrome and bacterial superantigens: an update. Annu. Rev. Microbiol. **55:**77–104.
19. **Miethke, T., C. Wahl, K. Heeg, B. Echtenacher, P. H. Krammer, and H. Wagner.** 1992. T cell-mediated lethal shock triggered in mice by the superantigen SEB: critical role of TNF. J. Exp. Med. **175:**91–98.
20. **Milano, S., F. Arcoleo, P. D'Agostino, and E. Cillari.** 1997. Intraperitoneal injection of tetracyclines protects mice from lethal endotoxemia downregulating inducible nitric oxide synthase in various organs and cytokine and nitrate secretion in blood. Antimicrob. Agents Chemother. **41:**117–121.
21. **Parsonnet, J., R. K. Hickman, D. D. Eardley, and G. B. Pier.** 1985. Induction of human interleukin-1 by toxic shock syndrome toxin-1. J. Infect. Dis. **151:**514–522.
22. **Schlievert, P. M.** 1993. Role of superantigens in human disease. J. Infect. Dis. **167:**997–1002.
23. **Scholl, P., A. Diez, W. Mourad, J. Parsonnet, R. S. Geha, and T. Chatila.** 1989. Toxic shock syndrome toxin-1 binds to major histocompatibility complex class II molecules. Proc. Natl. Acad. Sci. USA **86:**4210–4214.
24. **Smith, G. N., L. P. Yu, K. D. Brandt, and W. N. Capello.** 1998. Oral administration of doxycycline reduces collagenases and gelatinase activities in extracts of human osteoarthritic cartilage. J. Rheumatol. **25:**532–535.
25. **Solomon, A., M. Rosenblatt, D. Q. Li, Z. Liu, D. Monroy, Z. Ji, B. L. Lokeshwar, and S. C. Pflugfelder.** 2000. Doxycycline inhibition of interleukin-1 in the corneal epithelium. Investig. Ophthalmol. Vis. Sci. **41:**2544–2557.
26. **Webster, G. F., S. M. Toso, and L. Hegemann.** 1994. Inhibition of a model of in vitro granuloma formation by tetracyclines and ciprofloxacin: involvement of protein kinase C. Arch. Dermatol. **130:**748–752.